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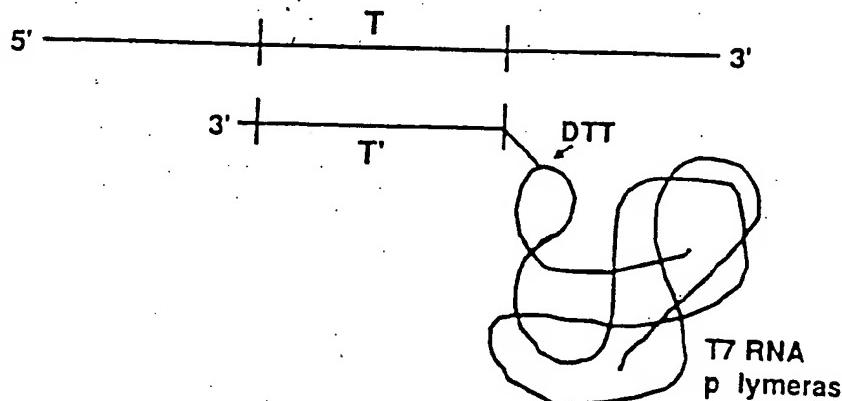
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(54) Title: REPLICATIVE RNA-BASED AMPLIFICATION DETECTION SYSTEMS		



(57) Abstract

This invention relates to the use of functional reporter molecules in the detection and measurement of nucleic acid sequences in a sample, as a determination, for example, of pathogenic disease existence or potential. The invention is predicated on the utilization of a transcription step between the production of an appropriate reporter molecule and replication based amplification in order to increase the number of detectable species as an indirect reference to target nucleic acid sequence.

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REPLICATIVE RNA-BASED AMPLIFICATION/DETECTION SYSTEMS

This is a continuing application of each of the applications U.S. Serial Nos. 07/241942 and 07/241969, each filed 8 September 1988.

Reference is made to U.S. Serial No. 852,692, 5 filed 16 April 1986, published as PCT International Application Publication No. WO 87/06270, and to its continuation-in-part application U.S. Serial No. 191,450, filed 9 May 1988, the entire disclosures of each of which are hereby incorporated by reference herein.

10 Field of the Invention

The present invention relates generally to advances in molecular biology and recombinant DNA technology.

More particularly, the present invention is 15 directed to the methods and means, including assays and pharmaceutical kits containing requisite reagents and means, for detecting in an in vitro or ex vivo setting the presence of nucleic acid species, and by deduction the corresponding polypeptide that nucleic acid encodes, 20 in a biological sample. The present invention makes use of replicative RNA for such detection and is predicated on the use of such replicative RNA to amplify by correspondence the segment of target nucleic acid or complement or hybridizing homologous segment. Thus, this 25 invention relates particularly to reporter systems that employ RNAs that serve as templates for self-replication catalyzed by RNA-dependent RNA polymerases.

Among the applications in which the present 30 invention finds use are in analyses of nucleic acid sequences characteristic of a particular or general pathogenic disease or condition by the in vitro or ex

vivo nucleic acid probe hybridization assays of body fluids and tissues containing requisite target nucleic acid.

Background of the Invention

5 It is a goal in this art to detect various nucleic acid sequences in a biological sample, in which the said sequences, as so-called target nucleic acid, is present in small amounts relative to its existence amongst a wide variety of other nucleic acid species  
10 including RNA, DNA or both. Thus, it is desirable to detect the nucleic acid encoding polypeptides that may be associated with pathological diseases or conditions, such as, for example, DNA correlating to that of the human immunodeficiency virus. In addition to the detection of  
15 nucleic acids encoding such viral particles, it is desirable to detect other nucleic acids characteristic of a pathological disease or condition such as a defective gene, as in the case of hemophilia, or in the detection of anti-pathogen antibodies of such diseases or  
20 conditions.

Characteristically, the nucleic acids associated with such are present, if at all, in very small amounts relative to total nucleic acid in a given biological sample, such as blood or other body fluid or  
25 tissue sample of a given individual to be tested.

Other important cases where the application of such technology finds use are detailed in said U.S. Serial Number 852,692 and need not be repeated here.

The detection of such nucleic acid species  
30 requires such specificity that, if present, it is detectable and measurable from amongst the wide variety of other nucleic acid species with which it is environmentally associated. Some of these species may bear close homology, at least in isolated segments, with  
35 the target nucleic acid. Further, as noted above, these

target nucleic acid species are very often found only in very minute amounts in the biological sample being tested. And yet, for proper diagnosis of the underlying disease state, it is essential that even small amounts of such target nucleic acid be detectable unequivocably for fidelity of the assay system.

Two fundamental approaches have been advanced for accomplishing the goal of the art. In one, the amount of nucleic acid in the sample is not altered or affected. Instead, a reporter system is developed whereby a large number of detectable molecules corresponding to the nucleic acid target are produced for ready detectability and measurement. Such a reporter system is a signal-generating system associated with the target nucleic acid producing a detectable signal representative of the number of molecules of target sequence. Such systems have employed a chromophore generating moiety linked to a oligonucleotide probe that hybridizes with the target nucleic acid sequence. The chromophore moiety can be isolated from those oligonucleotide probes that have properly hybridized to target, and measured. One such chromophore generating group is an enzyme such as alkaline phosphatase which has a chromogenic substrate producing under suitable conditions detectable and measurable colored molecules. Another such system employs radioactive labeling of the nucleic acid probe such that the signal generated by such properly hybridized target nucleic acid can be detected and measured.

A second approach has been developed that is fundamentally different in that it involves increasing the copy number of the target nucleic acid sequence itself, in particular in an amount greater than that of nucleic acid sequences with which it is associated in the sample. This can be done by selective amplification of the target nucleic acid sequence. One can refine the culture techniques of the sample such that somehow the

target nucleic acid sequence is amplified preferentially to other nucleic acid sequences. These techniques are cumbersome and time consuming and subject to trial and error.

5 Another example of the second approach is amplification of a target nucleic acid sequence in a so-called "polymerase chain reaction" (PCR). This technique was reported by Saiki et al., Science 230, 1350 (1985) and Mullis et al., European Patent Application  
10 Publication Nos. 200362 and 201184 (See also U.S. Patents 4683195 and 4683202), and particularly entails (1) hybridizing to a segment of target nucleic acid sequence a primer, (2) extending said primer with a polymerase, and (3) rendering single stranded the duplexes resulting  
15 from the chain extension reaction. This procedure can be repeated over a number of cycles so as to amplify the underlying target nucleic acid sequence. The procedure requires at least two nucleic acid probes and has three steps for a single cycle.

20 Certain RNAs are known to be susceptible to replication by certain polymerases, such as bacterial phage RNA-dependent RNA polymerase such as Q $\beta$  replicase and the replicase from brome mosaic virus (BMV). In this technique, the RNA can serve as a sequence template for replication by the RNA polymerase resulting in an amount  
25 of replicated RNA sequences that is an exponential increase of the amount initially present. See Miele et al., J. Molecular Biology 171, 281 (1983). A system in which probe for a target sequence is linked to an RNA capable of being replicated by Q $\beta$  replicase is described  
30 by Chu et al., Nucleic Acids Research 14, 5591 (1986) and by BMV replicase by March et al., Positive Strand RNA Viruses, Alan R. Liss (Publisher; New York) (1987; Proceedings of UCLA Symposium, 1986).

35 Until recently it has not been appreciated that (autocatalytic) replication could be employed to provide convenient, broadly applicable, highly sensitive reporter

systems for analyses of nucleic acid sequences. Above-cited U.S. Serial Number 852,692 provides the use of nucleic acid probe-replicative RNA adducts for use in detecting target nucleic acid sequences by amplification thereof via the exponential replicative process of the replicative RNA associated with the nucleotide probe.

5 Thus, that invention combines the art of replication of RNA with the use of oligonucleotide hybridization probes to detect target nucleic acid by associated replicative amplification. Details of that invention can be readily adduced by reference to the co-pending patent application or its counterpart, published international application, both cited supra. One practical drawback of that method resides in its necessary use of relatively long, hence 10 sensitive, sequences of replicatable RNA that may prove 15 inherently unstable in the assay environment.

It is an object of the present invention to take further advantage of the basic replicative process for amplification, for ease in the detection of sequences corresponding to target nucleic acid sequences. It is a further object of the present invention to take advantage of other biological processes that serve in result to achieve amplification of a given nucleic acid sequence. In particular, advantage is taken of the natural 20 transcription process (as the first step in expression of DNA to produce polypeptide products) whereby double-stranded nucleic acid templates containing a promoter sequence recognized by a DNA-dependent RNA polymerase is used to produce a plurality of corresponding RNA 25 transcripts. Again, using this process, a large number of RNA transcripts can be produced, that are themselves replicatable.

It is a further object of the present invention to combine the advantages of the replicative and transcript-producing procedures as a means for detecting 30 and measuring corresponding target nucleic acid.

It is thus an object of the present invention to produce, in all events, a given RNA transcript sequence that corresponds by presence and amount to target nucleic acid sequence and that can be replicated to a plurality and that can be adapted by association with a signal grouping that is accountable for its detection and measurement.

It is thus an overall object of the present invention to meet the goals enumerated by the art and to overcome the disadvantages and problems encountered by prior researchers' endeavors. The present invention utilizes, if at all, only relatively short, hence stable, RNA sequences that need only contain a sequence that insures replicability and nothing more. Thus, the present invention provides a straightforward technique that can be utilized with stable fidelity in an acceptably short period of time, employing the convenience of known reagents and having the precision necessary to reach consistent scientific results; one that can be employed in a reproducible assay setting and that is adaptable for use in kits for laboratory/clinical analyses. It is, hence, an object of the present invention to increase the detectability of certain nucleic acid sequences (target segments) by amplification of sequences associated with the presence of the target sequences in an in vitro or ex vivo system, utilizing the advantages provided by the natural transcription and replicative processes per se.

#### Summary of the Invention

The present invention is predicated on the use of an oligonucleotide probe, suitable for hybridization with a segment of a target nucleic acid sequence, that has linked thereto a moiety that is capable of initiating the production of a plurality of RNA transcripts, themselves containing sequence operable for their

multiple self-replication. The present invention thus employs novel adducts of covalently joined moieties, one an oligonucleotide probe capable of hybridizing with a target nucleic acid sequence and the other capable of initiating a transcription process producing a plurality of transcripts having the capability of self-replication.

In an embodiment, the present invention is directed to the novel adduct, its preparation and use, having linked moieties:

(1) an oligonucleotide sequence capable of hybridizing with a target nucleic acid sequence in a sample containing same; and

(2) a moiety capable of initiating a transcription process selected from the group consisting of an RNA polymerase and a nucleic acid promoter sequence.

Thus, in one aspect that embodiment is directed to the novel adduct, its preparation and use, having linked moieties:

(1) an oligonucleotide sequence capable of hybridizing with a target nucleic acid sequence in a sample containing same; and

(2) an RNA polymerase that, when optionally cleaved away, is capable of initiating transcription via a promoter sequence operably linked to DNA encoding replicatable RNA.

In a second aspect that embodiment is directed to the novel adduct, its preparation and use, having linked moieties:

(1) an oligonucleotide probe capable of hybridizing to a target nucleic acid sequence in a sample containing same; and

(2) a nucleic acid promoter sequence that, when optionally cleaved away, and when operably arranged with DNA encoding replicatable RNA, is recognized by a corresponding RNA polymerase to produce replicatable RNA transcripts.

The product RNA transcripts self-replicate with the aid of a suitable replicase and are then detected and measured in a manner known per se such as via an incorporating of, or association with, a chromophore moiety or a radioactively detectable moiety, for example.

In all respects, the present invention is directed to the novel application of the natural principles of transcript production, and their replication, for the deduced detection and measurement of corresponding target nucleic acid sequence that may be present in a biological sample containing a mixture of nucleic acids including DNA, RNA or both.

The present invention is thus directed to all methods and means associated with the preparation and use of replicatable RNA transcripts that can be amplified and detected as such and measured as a basis for the determination of the amount present, if any, of a corresponding target nucleic acid sequence. It is directed to their precursor adducts, that is, linked adducts of an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence and an RNA polymerase capable of recognizing a promoter, or a RNA polymerase recognizable promoter, operably linked to DNA encoding replicatable RNA. It is further directed to the preparation of such adducts and to their use in detecting by deduction a corresponding target nucleic acid sequence and measuring the amount of its presence in a given biological sample. The present invention is further directed to associated methods and means for devising assay systems based upon such adducts and their replicatable transcript products and to kits incorporating such assay methodology together with the necessary reagents and means for measuring target nucleic acid sequences in a laboratory/clinical setting.

The present invention thus reduces to a method useful for the detection of at least one specific nucleic

acid target sequence in a sample containing nucleic acid, comprising detecting self-replicated RNA transcript, it being the product of transcription of a molecule containing DNA encoding said replicatable RNA transcript operably linked to a promoter therefor, said promoter being susceptible to recognition by a polymerase reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence, or said promoter being a reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence.

The present invention primarily embodies 1) imposing a transcription step between the production of an appropriate reporter molecule and the replication event of amplification and 2) uses, if at all, relatively short, stable RNAs as reporter molecules. Necessarily, the replicability of the replicatable transcripts hereof follows by having disposed within the sequence of said transcripts a sequence that is recognized by replicase enzyme.

The present invention further embodies means for measuring the amount of said detected replicatable transcripts.

In an aspect, the present invention is directed to a method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising hybridizing under suitable conditions an oligonucleotide-RNA polymerase adduct comprising an oligonucleotide probe corresponding in sequence to a segment of said target sequence in a sample containing nucleic acid, and linked thereto a functional length of RNA polymerase, eliminating excess, non-hybridized oligonucleotide-RNA polymerase adduct, assaying the number of RNA polymerase sequences associated by hybridization with said target

nucleic acid sequence by using it to initiate transcription of a double-stranded DNA having a promoter recognizable by said RNA polymerase operably linked to a DNA sequence encoding replicatable RNA transcript,

5 allowing the transcript products to replicate, and detecting the replicated transcripts.

In an aspect, the present invention is directed to a method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising hybridizing under suitable conditions an oligonucleotide-promoter adduct comprising an oligonucleotide probe corresponding in sequence to a segment of said target sequence in a sample containing nucleic acid, and linked thereto a functional length of a strand of promoter sequence,

10 eliminating excess, non-hybridized oligonucleotide-promoter adduct,

15 assaying the number of promoter sequences associated by hybridization with said target nucleic acid sequence by using it to direct transcription of a single- or double-stranded DNA having the opposite strand of said promoter operably linked thereto, said DNA sequence encoding replicatable RNA transcript,

20 allowing the transcript products to replicate, and detecting the replicated transcripts.

The present invention, in application, embodies the detection of said self-replicated RNA transcripts such as via radio- or chromophore-labeling techniques known per se.

25 The present invention contemplates the detection of target nucleic acid sequence in a sample wherein said target nucleic acid sequence is associated with characteristics of a genetic or pathogenic disease or condition, and particularly those wherein the nucleic acid sequence is a segment of a human virus or is a segment of a defective gene.

There are a number of human diseases that are either the direct result of a genetic defect or are correlated with the presence of a particular genetic allele. By way of example, the technique described in 5 this application could be used to determine whether or not a given target gene is present in a very small sample of DNA. This would be useful in the prenatal diagnosis of genetic disorders such as hydrops fetalis (absence of  $\alpha$  globin DNA) or Lepore hemoglobinopathy (nonhomologous crossing over between  $\delta$  and  $\beta$  globin genes). The 10 technique could also be used to detect mRNA species. It would be useful, for example, in the diagnosis of Cooley's anemia; a disease characterized by the absence of  $\beta$  globin mRNA. Another potential application is the 15 detection of latent viral infections. DNA from peripheral blood cells could be tested for the presence of HIV-1 (AIDS virus) DNA which has become integrated into the host genome. The technique may also be used to determine the HLA type of a small tissue sample. This 20 would be useful in assessing the genetic predisposition of an individual to disorders such as ankylosing spondylitis and Reiter's syndrome.

The present invention contemplates the use of 25 particular promoters such as the bacteriophage T7 promoter and wherein RNA transcripts are produced using T7 RNA polymerase or use of the SP6 promoter and corresponding SP6 RNA polymerase.

The present invention is also directed to assay 30 systems and kits embodying same, useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising detecting self-replicated RNA transcript produced from a DNA molecule encoding same operably linked to a promoter therefor, said promoter being susceptible to recognition 35 by a polymerase reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence, or said promoter being

a reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence, and means for hybridizing said probe and utilizing the linked reporter of said hybridized probe to initiate or cause transcription of said DNA molecule and thereby to detect and measure said replicatable RNA transcript products therefrom, and by deduction said target sequence.

### Detailed Description of the Invention

10                   1. Brief description of the drawings

Figure 1 depicts schematically an aspect of this invention: a target nucleic acid sequence (T) having hybridized thereto a novel oligonucleotide-polymerase adduct hereof, the oligonucleotide moiety (T') being linked to the optionally cleavable--with dithiothreitol (DTT)--polymerase moiety (depicted as T7 RNA polymerase, conformationally).

Figure 2 depicts schematically an aspect of this invention: a target nucleic acid sequence (T) having hybridized thereto a novel oligonucleotide-promoter adduct hereof, the oligonucleotide moiety (T') being linked to the optionally cleavable promoter (P-) (depicted as a single strand).

## 2. General methods and definitions

25 Reference is made to standard textbooks of molecular biology that contain definitions and methods and means for carrying out basic techniques of the present invention, such as:

30 DNA probe or primer preparation, including DNA synthesis or isolation of sequences from natural source via restriction enzyme cleavage and the tailoring thereof so as to be suitable as such or when linked to other DNA for use as a primer or probe herein;

preparation of the linked adducts of oligonucleotides and nucleic acids or polypeptides for use in hybridization as oligonucleotide probe/reporter molecule;

5 hybridization methodology including variations in stringency conditions for producing more or less hybridization certainty depending on the degree of homology of the primer to a target DNA sequence;

10 identification, isolation or preparation of promoters, or more specifically promoters or sites recognized by bacteriophage DNA-dependant RNA polymerase and bacteriophage RNA-dependant RNA polymerase or in the employment of eukaryotic systems, viral DNA- and RNA-dependent RNA polymerases, for example, adenovirus  
15 encoded RNA polymerase and brome mosaic virus RNA polymerase;

identification, isolation or preparation of RNA polymerases capable of recognizing said promoters referred to above;

20 conditions conducive to the production of RNA transcripts, including so-called transcription-enhancer sequences;

the mechanism and methodology for (induced) replication; and so forth.

25 See, for example, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York 1982), and the various references cited therein; Hong, Bioscience Reports 1, 243 (1981); Cooke et al., J. Biol. Chem. 255 6502 (1980); and Zoller et al., Methods in Enzymology 100, 468-500 (1983); Crea et al., Nucleic Acids Res. 8, 2331 (1980); Narang et al., Meth. Enzym. 68, 90 (1979); Beauchage et al., Tetrahedron Letters 22, 1859 (1981); Brown et al., Meth. Enzym. 68, 109 (1979); Caruthers et al., Meth. Enzym. 154, 287  
30 (1985); Hitzeman et al., J. Biol. Chem. 255, 2073 (1980); Lee et al., Science 239, 1288 (1988); Milligan et al., Nucleic Acids Res. 15, 8783 (1987); Miller et al.,

5           Virology 125, 236 (1983); Ahlquist *et al.*, J. Mol. Biol. 153, 23 (1981); Miller *et al.*, Nature 313, 68 (1985); Ahlquist *et al.*, J. Mol. Biol. 172, 369 (1984); Ahlquist *et al.*, Plant Mol. Biol. 3, 37 (1984); Ou *et al.*, PNAS 79, 5235 (1982); Chu *et al.*, Nucl. Acids Res. 14, 5591 (1986); European Patent Application Publn. No. (EPA) 194809; Marsh *et al.*, Positive Strand RNA Viruses, p. 327-336, Alan R. Liss (publ.; New York) (1987); Proceedings of UCLA Symposium, 1986); Miller *et al.*, J. Mol. Biol. 187, 537 (1986); Stoflet *et al.*, Science 239, 491 (1988); Kramer *et al.*, J. Mol. Biol. 89, 719 (1974); Saris *et al.*, Nucl. Acids Res. 10, 4831 (1982); Bresser *et al.*, PNAS 80, 6523 (1983); and Chu *et al.*, Nucleic Acids Research 16, 3671 (1988), as well as the references cited therein.

10           All of the aforecited publications are by this reference hereby incorporated by reference herein.

15           By the term "promoter" is meant a nucleic acid sequence (naturally occurring or synthetically produced or a product of restriction digest) that is specifically recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription whereby an RNA transcript is produced. It may optionally contain nucleotide bases extending beyond the actual recognition site, thought to impart additional stability toward degradation processes, and may also include additional plus (+) nucleotides contiguous to the transcription initiation site. In principle, any promoter sequence may be employed for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Typical, known and useful promoters are those that are recognized by certain bacteriophage polymerase such as bacteriophage T3, T7 or SP6. See Siebenlist *et al.*, Cell 20, 269 (1980). These are but examples of those polymerases that can be employed in the practice of the present invention in conjunction with their associated promoter sequences.

As the promoter, in one aspect hereof, is the reporter molecule, it is defined, because it exists as a single-stranded version of an otherwise fully operable, classically defined, double-stranded promoter as given immediately above.

The "RNA transcript" hereof is the ribonucleic acid sequence produced after transcription initiation following RNA polymerase recognition of the promoter sequence (See supra). The production of such transcripts is more or less continuous, dependent in part on the amount of polymerase present.

By the term "primer" in the present context is meant a nucleic acid sequence (naturally occurring or synthetically produced or a product of restriction digest) that has sufficient homology with the target sequence such that under suitable hybridization conditions it is capable of hybridizing, that is binding to, the target sequence. A typical primer is at least about 10 nucleotides in length, and most preferably is of approximately 35 or more nucleotide bases in length, and in its most preferred embodiments, it shares identity or very high homology with the target sequence. See, for example, EPA 128042 (publd. 12 Dec 84).

The term "operably linked" in particular in connection with the linkage of a promoter sequence within an RNA encoding DNA sequence, refers to its functionality in producing corresponding RNA transcripts when the promoter is recognized by the suitable polymerase--see supra.

The techniques of forming a detection signal such as via radioactive labeling or chromogenic means using a chromogenic susceptible enzyme are also well known and documented in the art.

A sample on which the assay method of the invention is carried out can be a raw specimen of biological material, such as serum or other body fluid, tissue culture medium or food material. More typically,

the method is carried out on a sample which is a processed specimen, derived from a raw specimen by various treatments to remove materials that would interfere with detection of target, such as by causing non-specific binding of affinity molecules. Methods of processing raw samples to obtain a sample more suitable for the assay methods of the invention are well known in the art.

Thus, the method can be carried out on nucleic acid from cells following the colony hybridization method of Grunstein *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) **72**, 3961 (1975) (see also, U.S. Patent Nos. 4,358,535 and 4,562,159) or the plaque lift method of Benton *et al.*, Science **196**, 180 (1977). It can also be carried out on nucleic acids isolated from viroids, viruses or cells of a specimen and deposited onto solid supports (Gillespie *et al.*, J. Mol. Biol. **12**, 829 (1965)); including solid supports on dipsticks and the inside walls of microtiter plate wells. The method can also be carried out with nucleic acid isolated from specimens and deposited on solid support by "dot" blotting (Kafatos *et al.*, Nucl. Acids Res. **7**, 1541 (1979); White *et al.*, J. Biol. Chem. **257**, 8569 (1982); Southern blotting (Southern, J. Mol. Biol. **98**, 503 (1975)); "northern" blotting (Thomas, Proc. Natl. Acad. Sci. (U.S.A.) **77**, 5201 (1980); and electroblotting (Stellwag et al., Nucl. Acids Res. **8**, 299 (1980))). Nucleic acid of specimens can also be assayed by the method of the present invention applied to water phase hybridization (Britten et al., Science **161**, 527 (1968)) and water/organic interphase hybridizations (Kohne et al., Biochemistry **16**, 5329 (1977)). Water/organic interphase hybridizations have the advantage of proceeding with very rapid kinetics but are not suitable when an organic phase-soluble linking moiety, such as biotin, is joined to the nucleic acid affinity molecule.

5           The assay method of the invention can also be carried out on proteins or polysaccharides isolated from specimens and deposited onto solid supports by dot-blotting, by "Western" blotting, or by adsorption onto walls of microtiter plate wells or solid support materials on dipsticks.

10          Still further, the method of the invention is applicable to detecting cellular proteins or polysaccharides on the surfaces of whole cells from a specimen or proteins or polysaccharides from microorganisms immobilized on a solid support, such as replica-plated bacteria or yeast.

15          Reference herein to bacteriophage Q $\beta$  is not limited to any particular variant or mutant or population thereof. Such reference, unless otherwise specifically limited, is to any variant, mutant or population which, upon infection therewith of E. coli susceptible to bacteriophage Q $\beta$  infection, is capable of causing production of an RNA-dependent RNA-polymerase.

20          For other phages which, upon infection of bacteria susceptible to infection therewith, produce RNA-dependent RNA polymerases, and associated replicatable RNAs capable of being autocatalytically replicated in vitro, which can be employed in the present invention, see, e.g., Miyake et al., Proc. Natl. Acad. Sci. (U.S.A.) 68, 2022 (1971).

25          The term "linked" herein referring to the moieties of the adduct contemplates both covalent and non-covalent bonding, preferably covalent.

30          Examples of covalent linkages include, among others, the following:

35          (a) Linking moiety is a phosphate group and linkage is directly between the phosphate and the 5'-carbon of the 5'-nucleotide of promoter oligonucleotide. The phosphate linking moiety, bonded to the 5'-carbon of the 5'-nucleotide of promoter oligonucleotide, will usually be involved in covalently

joining a promoter oligonucleotide directly to the 3'-carbon of the 3'-nucleotide of a nucleic acid or to the 3'-carbon of the 3'-nucleotide of a segment of nucleotides which is a linking moiety considered to be bonded to the 3'-end of a nucleic acid molecule and which is covalently joined, through a phosphate at the 5'-carbon of its 5'-nucleotide, to the 3'-carbon of the 3'-nucleotide of the affinity molecule. Alternatively, the adduct hereof having a promoter can be wholly synthesized by methods known generally in the art.

(b) Linking moiety is biotinyl or iminobiotinyl and linkage is to the 5'-carbon of the 5'-nucleotide of promoter oligonucleotide through a spacer group of formula  $-\text{NH}(\text{CH}_2)_{\text{aa}}\text{NH}(\text{PO}_2)\text{O}-$ , formula  $-\text{NH}(\text{CH}_2)_{\text{bb}}\text{SS}(\text{CH}_2)_{\text{cc}}\text{NH}(\text{PO}_2)\text{O}-$ , or formula  $-\text{HN}(\text{CH}_2)_{\text{bb}}(\text{CO})(\text{NH})(\text{CH}_2)_{\text{cc}}\text{NH}(\text{PO}_2)\text{O}-$  wherein, in each case, the phosphoramidate group is bonded to the 5'-nucleotide and the amino group to the biotinyl or iminobiotinyl, aa is 2 to 20, and bb and cc are the same or different and are each 2 to 10. Promoter oligonucleotide with spacer group of formula  $-\text{NH}(\text{CH}_2)_{\text{aa}}\text{NH}(\text{PO}_2)\text{O}-$  can be made following the teaching of Chu and Orgel, DNA 4, 327 (1985). Replicative RNA with spacer group of formula  $-\text{NH}(\text{CH}_2)_{\text{bb}}\text{SS}(\text{CH}_2)_{\text{cc}}\text{NH}(\text{PO}_2)\text{O}-$  is taught in Example I. Promoter oligonucleotide with spacer group of formula  $-\text{NH}(\text{CH}_2)_{\text{bb}}(\text{CO})(\text{NH})(\text{CH}_2)_{\text{cc}}\text{NH}(\text{PO}_2)\text{O}-$  is made by reacting promoter oligonucleotide with group of formula  $-\text{O}(\text{PO}_2)\text{NH}(\text{CH}_2)_{\text{cc}}\text{NH}_2$  bonded to the 5'-carbon of the 5'-nucleotide, with an active ester of the aminocarboxylic acid of formula  $\text{NH}_2(\text{CH}_2)_{\text{bb}}\text{CO}_2\text{H}$ . Reaction of N-hydroxysuccinimo ester of biotin or iminobiotin to form a biotin-amide or iminobiotin-amide linkage with a primary amino group is known in the art.

(c) An amino group linking moiety linked through a spacer group of formula  $-(\text{CH}_2)_{\text{aa}}(\text{NH})(\text{PO}_2)\text{O}-$  or  $-(\text{CH}_2)_{\text{bb}}\text{SS}(\text{CH}_2)_{\text{cc}}\text{NH}(\text{PO}_2)\text{O}-$ , wherein the phosphoramidate group is linked to the 5'-carbon of the

5'-nucleotide of the promoter oligonucleotide and wherein aa, bb and cc are as defined supra. The methods of Chu and Orgel, DNA 4, 327 can be employed to prepare such promoter oligonucleotide.

5 (d) A sulfur linking moiety joined by a spacer group of formula -(CH<sub>2</sub>)<sub>cc</sub>NH(PO<sub>2</sub>)O-, wherein the phosphoramidate group is bound to the 5'-carbon of the 5'-nucleotide of promoter oligonucleotide and cc is as defined above. See Chu and Orgel, Nucleic Acids Research 16, 3671 (1988).

10 Among additional information in the art relating to joining linking moieties to proteins and nucleic acids see, e.g., Dreyer et al., Proc. Natl. Acad. Sci. (U.S.A.) 82, 968 (1985); Forster et al., Nucl. Acids Res. 13, 745 (1984); Ward et al., European Patent Application Publication No. 0 063 879; Englehardt et al., European Patent Application Publication No. 0 097 373; Alagon et al., Biochemistry 19, 4341 (1980); Imam et al., Cancer Res. 45, 263 (1985); and especially, Chu et al., Nucleic Acids Research 16, 3671 (1988).

15 20 The replicated transcripts (RNA) can be detected in a number of different ways:

25 Detection can be by ultraviolet absorbance of replicated RNA, as, for example, by the method of contact photoprinting (Kutateladze et al., Anal. Biochem. 100, 129 (1979)).

30 By employing a radioactively labeled ribonucleoside-5'-triphosphate in the replication reaction (e.g., <sup>3</sup>H-labeled or alpha-<sup>32</sup>PO<sub>4</sub>-labeled), so that the replicated RNA is radioactive, the replicated RNA can be detected, by any of numerous known procedures, by means of its radioactivity.

35 Biotin or iminobiotin can be incorporated into replicated RNA, which can then be detected by known techniques with an enzyme-avidin or enzyme-streptavidin adduct, which binds to the RNA-bound biotin and catalyzes production of a conveniently detectable chromogen.

Incorporation of biotin or iminobiotin can be accomplished by employing UTP that is biotinylated through a spacer to carbon-5 of the uracil moiety as a substrate for the replicase in the replication reaction.

Such UTP's are known compounds. Further, it is known that such UTP's are substrates for Q $\beta$  replicase, and that RNAs which include uracils biotinylated through spacer groups joined to the carbon-5 position, due to use of such UTP's in their synthesis, are templates for Q $\beta$  replicase catalyzed replication.

RNA resulting from the replication process could also be biotinylated employing photobiotin acetate and then detected, with an avidin-enzyme adduct-chromogenic compound system, like replicated RNA's synthesized with biotinylated UTP in the replication reaction.

RNA resulting from the replication process can be made fluorescent by employing a T4 RNA ligase catalyzed reaction to append nucleotides modified to be fluorescent to the 3'-end of replicative RNA. See Cosstick *et al.*, Nucl. Acids Res. 12, 1791 (1984). The fluorescence of the resulting RNA can be employed to detect the RNA by any of several standard techniques.

Among still other methods that can be used to detect replicated RNA are those wherein a reporter substance, that binds specifically with nucleic acid, is added to the system in which the replication has taken place, or to the medium, such as a positively charged support such as ECTEOLA paper, on which replicated RNA has been isolated, and signal from the reporter substance measured. Such substances include: chromogenic dyes, such as "stains all" (Dahlberg *et al.*, J. Mol. Biol. 41, 139 (1969); methylene blue (Dingman *et al.*, Biochemistry 7, 659 (1968), and silver stain (Sammons *et al.*, Electrophoresis 2, 135 (1981); Igloi, Anal. Biochem. 134, 184 (1983)); fluorogenic compounds that bind to RNA -- for example, ethidium bromide

(Sharp *et al.*, Biochemistry 12, 3055 (1973); Bailey *et al.*, Anal. Biochem. 70, 75 (1976); and fluorogenic compounds that bind specifically to RNAs that are templates for replication by Q $\beta$  replicase -- for example, 5 a phycobiliprotein (Ol *et al.*, J. Cell Biol. 93, 981 (1982); Stryer *et al.*, U.S. Patent No. 4,520,110) conjugated to the viral subunit of Q $\beta$  replicase.

Provided that the concentration of replicase remains above the concentration of template RNA, and that 10 ribonucleoside-5'-triphosphate concentration does not become limiting, the concentration of template RNA will increase exponentially with time during replicase-catalyzed RNA replication. After template RNA concentration equals or exceeds replicase concentration, 15 as long as ribonucleoside-5'-triphosphate concentration does not become limiting, the concentration of template RNA will increase linearly with time. See, e.g., Kramer *et al.* (1974), supra.

It has been found that, under the conditions 20 for replicase-catalyzed replication, the MDV-1 RNA there exemplified doubled in concentration every 36 seconds, until template concentration exceeded enzyme concentration.

The concentration of template RNA, in a 25 replicase-catalyzed replication reaction system after a given time for reaction, will be related to the initial concentration of template RNA. If, at all times during the replication reaction, the concentration of replicase exceeds that of template (and ribonucleoside-5'-triphosphate concentration does not become limiting), the log of concentration of template RNA at the conclusion of 30 the reaction will be directly proportional to the log of the initial concentration of template (at the start of the reaction). After replicase concentration falls below 35 template concentration, as long as ribonucleoside-5'-triphosphate concentration does not become limiting, the concentration of template at the

conclusion of reaction is directly proportional to the log of the initial concentration of template. Further, the time required for a reaction to reach the point at which template concentration equals replicase 5 concentration is proportional to the negative log of the initial concentration of template.

By allowing the replication reaction to proceed for longer times, greater sensitivity can be achieved.

In assays according to the invention, assays 10 are carried out simultaneously, under conditions as nearly alike as possible, on both test samples, which are being tested for analyte, and control samples. As understood in the art, control samples are similar to test samples but are known to contain either no analyte 15 or a known quantity of analyte. A control with no analyte establishes the "background," below which it is not possible to distinguish samples which contain analyte from those which do not. By comparing the amount or concentration of replicated replicative RNA produced in 20 an assay of a test sample with the amount or concentration produced with control samples assayed simultaneously, the presence of analyte in test sample at a level above background can be determined. If control samples with a range of known concentrations of analyte 25 are employed, the concentration of analyte in a test sample can be estimated.

Again, the use of a "replicase" for (autocatalytic) induction of replication of the RNA transcripts of the present invention are generally known 30 in the art. Suitable examples of such replicases that are useful in the present invention include the so-called Q $\beta$  virus replicase that recognizes certain nucleic acid sequence sites at both the 3'- and 5'- ends of the given RNA transcript and the so-called brome mosaic virus (BMV) 35 as well as the alpha virus replicases which are thought to recognize nucleic acid sequence sites at the 3' end of a given RNA transcript. These replicases serve to

replicate, that is reproduce, the RNA transcripts and complements so as to multiply copies thereof. When such enzyme is present in the reaction locus during the process of transcription, it can be foreseen that the multiple transcripts that are produced during transcription can themselves undergo replication so as to exponentially increase the amount of RNA transcript product.

10           3. Detailed description of particularly preferred embodiments

T7 RNA polymerase can be attached to an oligodeoxynucleotide probe via a cleavable linker. The target nucleic acid sequence contained in a sample is probed, and excess, non-hybridized oligonucleotide-T7 RNA polymerase adduct is displaced by washing. The T7 RNA polymerase is released by cleaving the linker under particularly preferred conditions. Determining the number of target molecules contained in the sample is achieved by assaying the T7 RNA polymerase molecules that are released. This is done by using the T7 RNA polymerase molecules to transcribe double-stranded DNA consisting of a T7 promoter joined to a DNA sequence that codes for an RNA substrate of Q $\beta$  RNA polymerase, for example MDV1 RNA. The replicable RNA is assayed using Q $\beta$  RNA polymerase in the manner set forth in co-pending patent application cited supra. The two foregoing enzymatic steps may preferably be carried out in a single pot reaction. The method of covalently linking the oligonucleotide probe to T7 RNA polymerase via a cleavable linker such that the enzyme is still active following the cleavage reaction can be in accordance with the method described by Chu et al., Nucleic Acids Research 16, 3671 (1988). It is also contemplated using a non-cleavable linker, releasing the hybridized probe-T7 RNA polymerase adduct into solution by thermal

denaturation. This method extends to antibody-dependent assays in an obvious way.

The target nucleic acid in a sample is probed using an oligodeoxynucleotide adduct that contains two subsequences: (1) a complement sequence of the target sequence, and (2) the appropriate single strand (minus strand) of the promoter for T7 RNA polymerase. Excess non-hybridized adduct is displaced by washing.

Hybridized material is then released from the target nucleic acid by simple denaturing and/or by displacement using an oligodioxinucleotide with greater affinity for the target. Alternatively, if the two subsequences are joined by a cleavable linker (for example, a disulfide bond), the portion that is complementary to the T7 promoter can be released by chemical methods, leaving the remaining portion bound to the target.

The released DNA that contains the complement of the T7 promoter serves as a reporter molecule for successful hybridization events. This DNA is hybridized to a single-stranded DNA molecule that contains the (plus) strand of the T7 promoter joined to a sequence that codes for an RNA substrate of Q $\beta$  RNA polymerase. The hybridization product is a functional double-stranded T7 promoter joined to a single-stranded template encoding an RNA transcript. The T7 RNA polymerase binds to the double-stranded promoter and proceeds to transcribe the single-stranded template (see Milligan et al., Nucleic Acids Research 15, 8783 (1987)). The resulting RNA is assayed using Q $\beta$  RNA polymerase exactly as is described in the patent application cited supra.

#### 4. Examples

##### Reference Example 1

Exemplified is the use of T7 RNA polymerase and Q $\beta$  RNA polymerase to amplify a signal generated by successful target hybridization events. T7 RNA

polymerase is a DNA-dependent RNA polymerase that has the following useful properties:

- (1) it initiates specifically at sites that lie adjacent to the T7 promoter;
- 5 (2) once initiation has occurred, the enzyme can operate on either single- or double- stranded templates;
- 10 (3) the enzyme has a high turnover rate, producing 200-1200 moles of RNA transcript per mole of DNA template;
- 15 (4) the gene for T7 RNA polymerase has been cloned, making it relatively straightforward to prepare very large amounts (2-10 MU) of the enzyme.

All class III (high efficiency) promoters of the T7 viral genome have a common 20 base-pair sequence from -17 to +3:

3'-ATTATGCTGAGTGATATCCC-5'

5'-TAATACGACTCACTATAGGG-3'

Beyond position +3 the template may exist as a single strand without adversely affecting transcription efficiency. Synthesis begins with the sequence GGG and proceeds in the 5'-->3' direction. The template is designed such that the product of transcription is the (+) strand of MDV-1 RNA. MDV-1 (+) RNA contains 221 nucleotides, beginning with the sequence GGG at its 5' end. It in turn serves as an ideal substrate for Q $\beta$  RNA polymerase, an RNA-dependent RNA polymerase which carries out autocatalytic amplification of its substrate RNA. Combining the T7 RNA polymerase system with the Q $\beta$  RNA polymerase system provides an extremely powerful tool for amplifying the signal generated by a rare molecular event.

#### Example 2

The target sequence 5'-GTTGTGTGGAAT-3' (T)  
35 which is part of the sequence of M13mp8(+) strand DNA is

detected. T', the complement of T, is linked to T7 RNA polymerase via a cleavable disulfide linkage, probe for the target with this probe-enzyme adduct, wash away excess adduct, release the T7 RNA polymerase from the probe and use it to transcribe double-stranded DNA consisting of T7 promoter joined to DNA that codes for MDV-1.

The target sequence 5'-GTTGTGTGGAATTGTG-3' (T) which is part of the sequence of the M13mp8 (+) strand 10 DNA is detected. T', the complement of T, is linked to the minus strand of the promoter and uses the general principle of the invention to detect T by detecting the production of MDV-1 RNA produced in a suitable assay.

The complement of the target (probe) 5'-CACAAATTCCACACAAAC-3' (sequence 1) and the minus strand of the promoter 5'-TAATACGACTCACTATAGGG-3' (sequence 2) is synthesized by the known, solid-phase phosphoamidite method, using a RNA synthesizer.

#### Preparation of Probe-T7 RNA Polymerase Adduct

The probe, 5'-ATTCCACACAAAC-3', synthesized by the known, solid-phase phosphoamidite method, is linked to T7 RNA polymerase via a disulfide bond using the procedure described (Chu *et al*, Nucleic Acids Research, 16, 3671 (1988)). T7 RNA polymerase is first thiolated 25 with iminothiolane and then reacted with 5'-2-(pyr)-SS-P-5'ATTCCACACAAAC to give the adduct:

T7 RNA polymerase-SS-CH<sub>2</sub>CH<sub>2</sub>-P-5'-ATTCCACACAAAC.

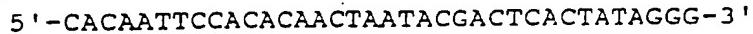
The procedure for the thiolation of T7 RNA polymerase is identical to the procedure described in Example 3 for the thiolation of anti-rubella IgG. 5'-(2-pyr)-SS-P-5'-ATTCCACACAAAC is synthesized exactly as described in Example 3 for making the 5'-(2-pyr)-SS-5'-P-promoter complement.

The target sequence 5'-GTTGTGTGGAATTGTG-3' (T) 35 which is part of the sequence of the M13mp8 (+) strand DNA is detected. T', the complement of T, is linked to

T7 RNA polymerase and uses the general principle of the invention to detect T by detecting the production of MDV-1 RNA produced in a suitable assay.

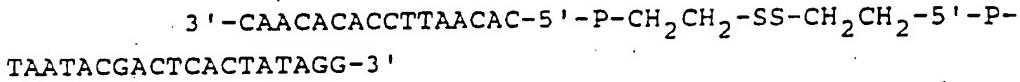
5 The linkage of probe to the minus strand of the promoter may be accomplished in two ways:

1) by normal phosphodiester linkage. The following sequence will be synthesized in a DNA synthesizer:



10 (sequence 3)

2) by a cleavable disulfide bond to form the following adduct:



15 The adduct is formed as described by Chu et al. Nucleic Acids Research 16, 3671 (1988). The sequences 1 and 2 above are converted to the 5'-phosphate derivatives using polynucleotide kinase and ATP. The 5'-phosphate derivatives (0.1-10 ODU) are converted to the 5'-cystamine derivatives by treatment with 0.1 M 1-methylimidazole, 0.15 M 1-ethyl-3,3-dimethylaminopropyl carbodiimide and 0.5 M cystamine at pH 7 and 50°C for 2 hours. The 5'-cystamine derivatives are purified either by HPLC on RPC-5 or denaturing gel electrophoresis.

25 A mixture containing 1  $\mu$ M of the 5'-cystamine derivative of sequence 2 and 5-8  $\mu$ M of the 5'-cystamine derivative of sequence 1 is treated with 5 mM DTT in TRIS-EDTA buffer at pH 7.2 for 1 hour at room temperature. The reaction mixture is then dialyzed against buffer containing 0.1 mM DTT, 1 mM Tris and 0.1 mM EDTA at pH 7.2 for 30 mins, and against fresh buffer containing 1 mM Tris and 0.1 mM EDTA at pH 7.2 for a further 30 mins. The mixture is then concentrated, if necessary in a speed-vac concentrator and the probe-promoter complement adduct purified by gel electrophoresis.

Preparation of Target Sample

M13mp8DNA (+) strand DNA (7229 bases), 1 fg, 10 fg, 100 fg, 1 pg, 10 pg, 100 pg ( $4 \times 10^{-7}$  fmole -  $4 \times 10^{-5}$  pmoles) is diluted to 200  $\mu$ l to give a final solution containing 10 mM Tris, 1 mM EDTA, 100 mM NaCl at pH 7.5. Then 20  $\mu$ l of 3 M NaOH are added and the solution is incubated for 30 mins at 60-70°C. After cooling, the solution is neutralized with 200  $\mu$ l of 2 M ammonium acetate pH 7.0. The DNA is slot-blotted onto nitrocellulose paper that has been pre-wetted with water and mM ammonium acetate using a manifold slot blotter. The paper is then baked in a vacuum oven at 80°C for 1 hour.

Hybridization and Release

Nitrocellulose blots containing M13mp8(+) strand DNA are pre-hybridized for 1 hour at 25°C in hybridization buffer (900 mM NaCl, 6mM EDTA, 90 mM Tris, pH 7.5, 0.1% SDS) containing 100  $\mu$ g/ml randomly cleaved RNA. Hybridization with 1  $\mu$ g/ml of the probe-T7 RNA polymerase adduct is then carried out for 10 minutes at 25°C. The blots are washed twice with buffer containing 180 mM NaCl and 18 mM NaCl. T7 RNA polymerase is released from the blot by incubation of the blot slot with 30  $\mu$ l of 10 mM DTT in Tris-EDTA buffer for 30 minutes. The released polymerase is then added to 20  $\mu$ l of a solution containing 1 pmole of double-stranded T7 promoter linked to DNA coding for MDV-1 RNA, 0.5  $\mu$ g of Q $\beta$  RNA polymerase, 12 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM DTT and 50 mM Tris, pH 7.5. 10  $\mu$ l of a solution containing 1 mM each of the four dNTP's is then added, bringing the total volume to 60  $\mu$ l. The combined mixture is incubated at 37°C for 20 minutes and then assayed for the production of MDV-1.

The nitrocellulose blots are pre-hybridized for 1 hour at 30°C in hybridization buffer (900 mM NaCl, 6 mM

EDTA, 90 mM Tris pH 7.5, 0.1% SDS) containing 100 µg/ml randomly cleaved RNA. Hybridization with 1 ng/ml of the probe-promoter complement adduct is then carried out at 45°C for 1 hour. The blots are then washed twice with buffer containing 180 mM NaCl at room temperature and again with buffer containing 18 mM NaCl at 30°C.

The probe promoter complement linked by phosphodiester bonds will be released from the target slots in 30 µl boiling buffer, cooled at room temperature for 15 mins. The probe linked to the promoter complement by disulfide bonds will be released by incubation of the target slot with 30 µl of 10 mM DDT in Tris-EDTA buffer at 37°C for 1 hour.

#### Hybridization of the Released Promoter

The released DNA, which contains the minus strand of the T7 promoter, serves as a reporter for successful target hybridization events. This DNA is in turn hybridized to a single-stranded DNA molecule which contains the 17-nucleotide plus (+) strand of the T7 promoter joined to a 221-nucleotide sequence which codes for MDV-1 RNA. Hybridization occurs in a 40 µl volume which contains 1 pmole (~100 ng) T7 promoter-MDV-1 DNA, 12 mM MgCl<sub>2</sub>, 2 mM spermidine, and 50 mM Tris (pH 7.5). This mixture is heated to 65°C for 5 min and then cooled to 30°C over 5-10 min. 20 µg (~100U) T7 RNA polymerase, 0.5 µg Qβ RNA polymerase, 12 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM DTT, 50 mM Tris (pH 7.5), and 1 mM each of the four NTPs is added, bringing the total volume to 60 µl. The combined mixture is incubated at 37°C for 20 min, and then assayed for the production of MDV-1 RNA.

#### Detection of Replicated RNA

The amount of RNA is determined by its intrinsic UV absorbance (e.g. as by the contact photoprinting method of Kutateladze *et al.*, Anal.

5           Biochem. 100, 129 (1979)). Alternatively, the RNA is visualized on ETEOLA paper. Aliquots (of equal volume) of replication reaction are transferred with 13, 48 or 96-fingered aliquotter to sheets of diethylaminoethyl cellulose paper. The sheets are then washed at room temperature in a solution of 200 mM NaCl, 300 mM ammonium acetate pH 6 to remove ribonucleosides not incorporated into RNA. The sheets are then stained with 0.3 µg/ml of ethidium bromide. (Sharp et al., Biochemistry 12, 3055  
10           (1973); Bailey et al., Anal. Biochem 70, 75 (1976).

Finally the fluorescence from individual blots is measured by any of several known techniques. Fluorescence intensity from a stained blot above that from control blots indicates the presence of analyte. 15 Other staining materials can be employed in place of ethidium bromide. These include methylene blue (Dingman and Peacock, Biochemistry 7, 659 (1968)), silver stain (Sammons, et al., Electrophoresis 2, 135 (1981)) or phycobiliprotein Q $\beta$  replicase conjugate (Oi et al., J. Cell Biol. 93, 981 (1982)) .  
20

#### Example 3

25           Rubella antibody is detected in a patient with recent exposure to rubella antigen. Microliter wells coated with rubella antigen are incubated for 3 hours at room temperature with 50 µl aliquots per well of 1:10, 1:30, 1:100, 1:300, 1:1000, and 1:3000 dilutions of IgG isolated from the patient. Dilutions are prepared with 5% horse serum in phosphate-buffered saline. The plates are then thoroughly washed with Tween 20-NaCl. To each 30 well is then added 50 µl of a solution containing 1 µg/ml of anti-rubella IgG linked by disulfide bonds to the T7 RNA polymerase. After 2 hours' incubation at room temperature, the plates are washed 3 times with NaCl-Tween 20. A solution of 30 µl of 100 mM DDT in Tris-EDTA Buffer is then added to the wells and incubated at room  
35

temperature for 1 hour. The released T7 RNA polymerase is then assayed as described in Example 2.

The synthesis of the anti-rubella IgG-T7 RNA polymerase adduct linked by disulfide bonds is carried out as described in USSN 852692 and King *et al.*, Biochemistry 17, 1499 (1978). Anti-rubella IgG is first thiolated with imino-thiolane and then reacted with, (2-pyr)-SS-T7 RNA polymerase to give the disulfide linked adduct:

10                   IgG-SS-T7 RNA polymerase.

In the preparation of the thiolated anti-rubella IgG, 200 m grams\* of rubells anti-IgG is reacted with 1 mM iminothiolane in buffer containing 60 mM triethylamine, 7 mM phosphate, 100 mM NaCl and 1 mM EDTA at pH 8 and 0°C for 1 hour (Blattler *et al.*, Biochemistry 24, 1517 (1985)). The thiolated protein is separated from unreacted iminothiolane by gel filtration and stored under nitrogen.

20                   400 µl of a solution containing 4.5 µM of the thiolated anti rubella IgG and 9.0 µM of the (2-pyr)-SS-T7 RNA polymerase is reacted at 25°C in 0.1 M Na<sub>2</sub>(PO<sub>4</sub>) buffer containing 1mM EDTA at pH 6.6 for 17 hours. It is then applied to a Biogel P-300 column and eluted with 0.1 M Na<sub>2</sub>(PO<sub>4</sub>) buffer (pH 7.0). The condensation product is separated from unreacted starting materials on the basis of size.

Example 4

30                   Rubella antibody is detected in a patient with recent exposure to rubella antigen. Microtiter wells coated with rubella antigen are incubated for 3 hours at room temperature with 50 µl aliquots per well of 1:10, 1:30, 1:100, 1:300, 1:1000, and 1:3000 dilutions of IgG isolated from the patient. Dilutions are prepared with 5% horse serum in phosphate-buffered saline. The plates are then thoroughly washed with Tween 20-NaCl. To each well is then added 50 µl of a solution containing 1 µg/ml

of anti-rubella IgG linked by disulfide bonds to the minus strand of the promoter. After 2 hours' incubation at room temperature, the plates are washed 3 times with NaCl-Tween 20. A solution of 30  $\mu$ l of 100 mM DDT in 5 Tris-EDTA Buffer is then added to the wells and incubated at room temperature for 1 hour. The released minus strand of the promoter is then assayed as described in Example 2.

The synthesis of the anti-rubella IgG-promoter minus strand adduct linked by disulfide bonds is carried out as described in USSN 852692, supra. Anti-rubella IgG is first thiolated with imino-thiolane and then reacted with the 5'-(2-pyr)-SS-P-sequence 2 to give the disulfide linked adduct:

15           IgG-SS-CH<sub>2</sub>CH<sub>2</sub>-P-5'-promoter complement

200  $\mu$ g of rubella anti-IgG is reacted with 1 mM iminothiolane in buffer containing 60 mM triethylamine, 7 mM phosphate, 100 mM NaCl and 1 mM EDTA at pH 8 and 0°C for 1 hour. (Blattler *et al*, Biochemistry 24, 1517 20 (1985). The thiolated antibody containing 1 mole of thiol per mole of IgG is separated from unreacted iminothiolane by gel filtration and stored under nitrogen.

25           The 5'-cystamine adduct of sequence 2 (promoter minus strand) (0.01-1.0 ODU) is treated with 5 mM DDT in 10  $\mu$ l of Tris-EDTA buffer pH 7 for 1 hour at room temperature. 40  $\mu$ l of a 3 mM solution of 2,2'-pyridyl disulfide is then added. After 1 hour at room temperature the 5'-(2-pyr)-SS-promoter minus strand is 30 purified by gel electrophoresis.

35           400  $\mu$ l of a solution containing 0.01-1.0 ODU of the 5'-(2-pyr)-SS-P-promoter complement and 1  $\mu$ M of the thiolated anti rubella IgG is dialyzed against buffer containing 1 mM NaCl, 1 mM Tris and 0.1 mM EDTA at pH 7.2 for 1 hour. The solution is then concentrated to 10  $\mu$ l in a speed-vac concentrator, and allowed to stand overnight at room temperature. It is then applied to a

DEAE column. Unreacted IgG is eluted with 50 mM Tris at pH 7, and the IgG-promoter complement adduct is eluted with the same buffer containing 0.25 M NaCl. Unreacted oligonucleotide can be eluted with buffer containing 0.5  
5 M NaCl.

The foregoing description details more specific methods that can be employed to practice the present invention and represents the best mode contemplated. However detailed the foregoing may appear in text, it  
10 should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

What is claimed is:

1. An adduct comprising linked moieties:
  - (1) an oligonucleotide sequence capable of hybridizing with a target nucleic acid sequence in a sample containing same; and
  - (2) a moiety capable of initiating a transcription process selected from the group consisting of an RNA polymerase and a nucleic acid promoter sequence.
- 10 2. An oligonucleotide-RNA polymerase adduct according to Claim 1 comprising linked moieties:
  - (1) an oligonucleotide sequence capable of hybridizing with a target nucleic acid sequence in a sample containing same; and
  - (2) an RNA polymerase that, when optionally cleaved away, is capable of initiating transcription via a promoter sequence operably linked to DNA encoding replicatable RNA.
- 15 3. An oligonucleotide-promoter adduct according to Claim 1 comprising linked moieties:
  - (1) an oligonucleotide sequence capable of hybridizing with a target nucleic acid sequence in a sample containing same; and
  - (2) a nucleic acid promoter sequence that, when optionally cleaved away, and when operably arranged with DNA encoding replicatable RNA, is recognized by a corresponding RNA polymerase to produce replicatable RNA transcripts.
- 20 4. The adduct according to Claim 2 or 3 wherein said oligonucleotide sequence is a DNA segment corresponding to a human immunodeficiency virus.
- 25 5. The adduct according to Claim 2 or 3 wherein said oligonucleotide sequence is a segment of a defective gene.
- 30 6. The adduct according to Claim 2 or 3 wherein said RNA polymerase is T7 RNA polymerase.

7. The adduct according to Claim 2 or 3 wherein said RNA polymerase is SP6 RNA polymerase.

8. A method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising detecting self-replicated RNA transcript, said transcript being the product of transcription of a molecule containing DNA encoding said replicatable RNA transcript operably linked to a promoter therefor, said promoter being susceptible to recognition by a polymerase reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence, or said promoter being a reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence.

9. The method according to Claim 6 wherein said self-replicated RNA transcript is the means of amplification of deduced target sequence and a transcription step is imposed between the production of the reporter molecule detecting the presence of target sequence and the replication event of amplification.

10. A method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising

hybridizing under suitable conditions an oligonucleotide-RNA polymerase adduct comprising an oligonucleotide probe corresponding in sequence to a segment of said target sequence in a sample containing nucleic acid, and linked thereto a functional length of RNA polymerase,

eliminating excess, non-hybridized oligonucleotide-RNA polymerase adduct, assaying the number of RNA polymerase sequences associated by hybridization with said target nucleic acid sequence by using it to initiate transcription of a double-stranded DNA having a promoter

recognizable by said RNA polymerase operably linked to a DNA sequence encoding replicatable RNA transcript, allowing the transcript product to replicate, and

5 detecting the replicated transcripts.

11. A method useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising

hybridizing under suitable conditions an 10 oligonucleotide-promoter adduct comprising an oligonucleotide probe corresponding in sequence to a segment of said target sequence in a sample containing nucleic acid, and linked thereto a functional length of a strand of promoter sequence,

15 eliminating excess, non-hybridized oligonucleotide-promoter adduct,

assaying the number of promoter sequences associated by hybridization with said target nucleic acid sequence by using it to direct transcription of a single- 20 or double-stranded DNA having the opposite strand of said promoter operably linked thereto, said DNA sequence encoding replicatable RNA transcript,

allowing the transcript product to replicate, and

25 detecting the replicated transcripts.

12. The method according to Claim 10 or 11 wherein the detected replicated transcripts are measured in a standardized manner so as to measure the amount of target sequence contained in a sample of nucleic acid.

30 13. The method according to Claim 10 or 11 wherein said target sequence is disposed within a nucleic acid sequence associated with the characteristics of a genetic or pathogenic disease or condition.

14. The method according to Claim 13 wherein 35 said nucleic acid sequence is a DNA segment corresponding to a human immunodeficiency virus.

15. · The method according to Claim 13 wherein said nucleic acid sequence is a segment of a defective gene.
16. The method according to Claim 10 or 11 wherein said RNA polymerase is T7 RNA polymerase.
17. The method according to Claim 10 or 11 wherein said RNA polymerase is SP6 RNA polymerase.
18. The method according to Claim 10 or 11 wherein said detected replicated transcripts are labeled prior to detection.
19. The method according to Claim 18 wherein said transcripts are radio-labeled.
20. The method according to Claim 19 wherein said transcripts are chromophore labeled.
21. A kit useful for the detection of at least one specific nucleic acid target sequence in a sample containing nucleic acid, comprising detecting self-replicated RNA transcript produced from a DNA molecule encoding same operably linked to a promoter therefor, said promoter being susceptible to recognition by an RNA polymerase reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence, or said promoter being a reporter molecule associated as an adduct with an oligonucleotide probe capable of hybridizing with said target nucleic acid sequence, and means for hybridizing said probe and utilizing the linked reporter of said hybridized probe to initiate or cause transcription of said DNA molecule and thereby to detect and measure said replicatable RNA transcript products therefrom, and by deduction said target sequence.

1/1

FIG. 1

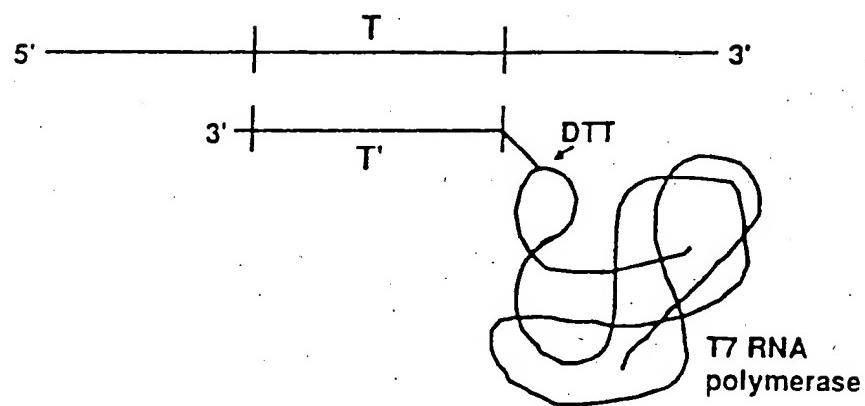
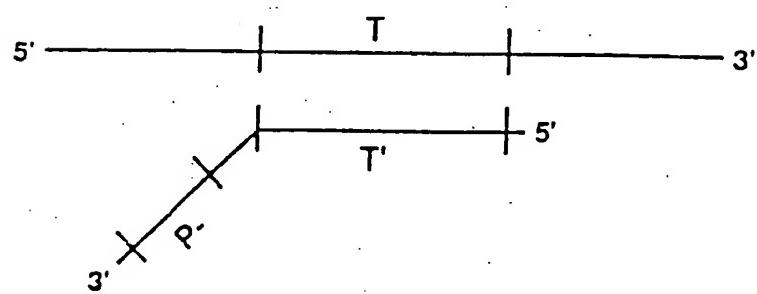


FIG. 2



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03833

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

**U.S. Cl.:** 435/6  
**IPC(4):** C12Q 1/68

## II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched <sup>7</sup>	
		Classification Symbols
U.S.	435/6	935/78,111.
	436/501	
	536/27	
		Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	US, A, 4,495,280 BUJARD ET AL. 22 January 1985, see the abstract.	1 and 3
X	US, A, 4,761,367 EDGELL ET AL. 2 August 1988, see the abstract.	1
P,X	US, A, 4,859,601 BURNETT, JR. ET AL. 22 August 1989, see the abstract.	1 and 3
X	WO, A, 87/06270 CHU 22 October 1987. See the entire document.	1-21

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date, or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

30 November 1989

International Searching Authority

SA/US

Date of Mailing of this International Search Report

18 DEC 1989

Signature of Authorized Officer

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